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The wound healing properties of *Channa striatus*-cetrimide cream-wound contraction and glycosaminoglycan measurement

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Abstract

Haruan has been proved to influence the different phases of wound healing process. The current research focuses on the effects of haruan on the different constituents of extracellular matrix of healing wounds in normal and diabetic rats. Anaesthetized normal and streptozotocin induced diabetic rats were provided with excision wounds at the back and then animals were divided into four groups as: group 1, wounds treated with cetrimide + haruan cream; group 2, wounds treated with haruan cream; group 3, wounds treated with cetrimide (commercial) cream; and group 4, wounds untreated and served as control. Animals were sacrificed after 3, 6, 9 and 12 days. These wounds were used to determine the hexosamine, protein, uronic acid and glycosaminoglycan contents and the wound contraction. The results suggested a marked increase ($P < 0.05$) in the uronic acid, hexosamine and dermatan sulfate contents on day 3 of group 1 when compared with groups 2–4. Wound contraction of group 1 was also markedly enhanced of group 1 ($P < 0.01$) when compared with groups 2– 4. On the basis of these results, we conclude that haruan enhances the synthesis of different glycosaminoglycans in healing wounds, which are the first component of extracellular matrix to be synthesized during the wound healing process. The enhanced levels of glycosaminoglycans may help in the formation of a resistant scar and enhanced wound contraction represents the positive influence of haruan on the fibroplastic phase of wound healing.

Keywords: Glycosaminoglycans; Wound contraction; Extracellular matrix

1. Introduction

Wound healing, a complex sequence of events is initiated by the stimulus of injury to tissues. A positive stimulus may result from the release of some factors by the wounding of tissues (Alison, 1992). This sequence of physiologic events occurs by a process of connective tissue repair. These events involve the migration, proliferation, adhesion and differentiation of the cells (Raghow, 1994). Glycosaminoglycans are the important constituents of the extracellular matrix found on

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cell surfaces and, are the first component of the extracellular matrix to be synthesized during wound healing process (Chithra et al., 1998).

Channa striatus (haruan) is being used in the belief that it enhances the wound healing after surgical operations and reduces the post-operative pain (Mat Jais et al., 1997). The fatty acid and amino acid composition of this fish is also well-established (Mat Jais et al., 1994). The previous studies (Baie and Sheikh, 1999) on the determination of tensile strength of healing wounds treated with *C. striatus*, made the present study more meaningful for the determination of biochemical components of healing wounds.

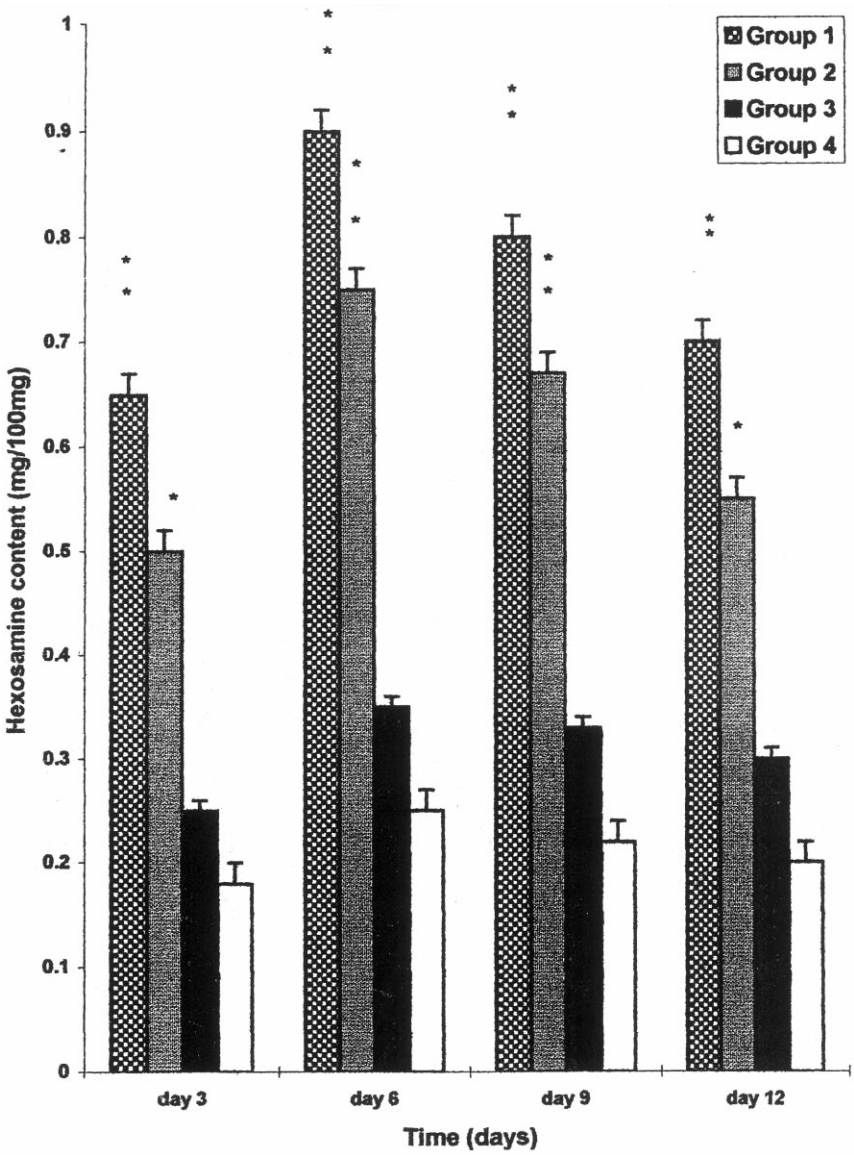

Fig. 1. Effect of different creams on the hexosamine content of normal healing wounds at various time intervals. Results are presented as means $\pm$ S.D. Statistically significant data are given as $^{*}P < 0.05$ and $^{**}P < 0.01$.

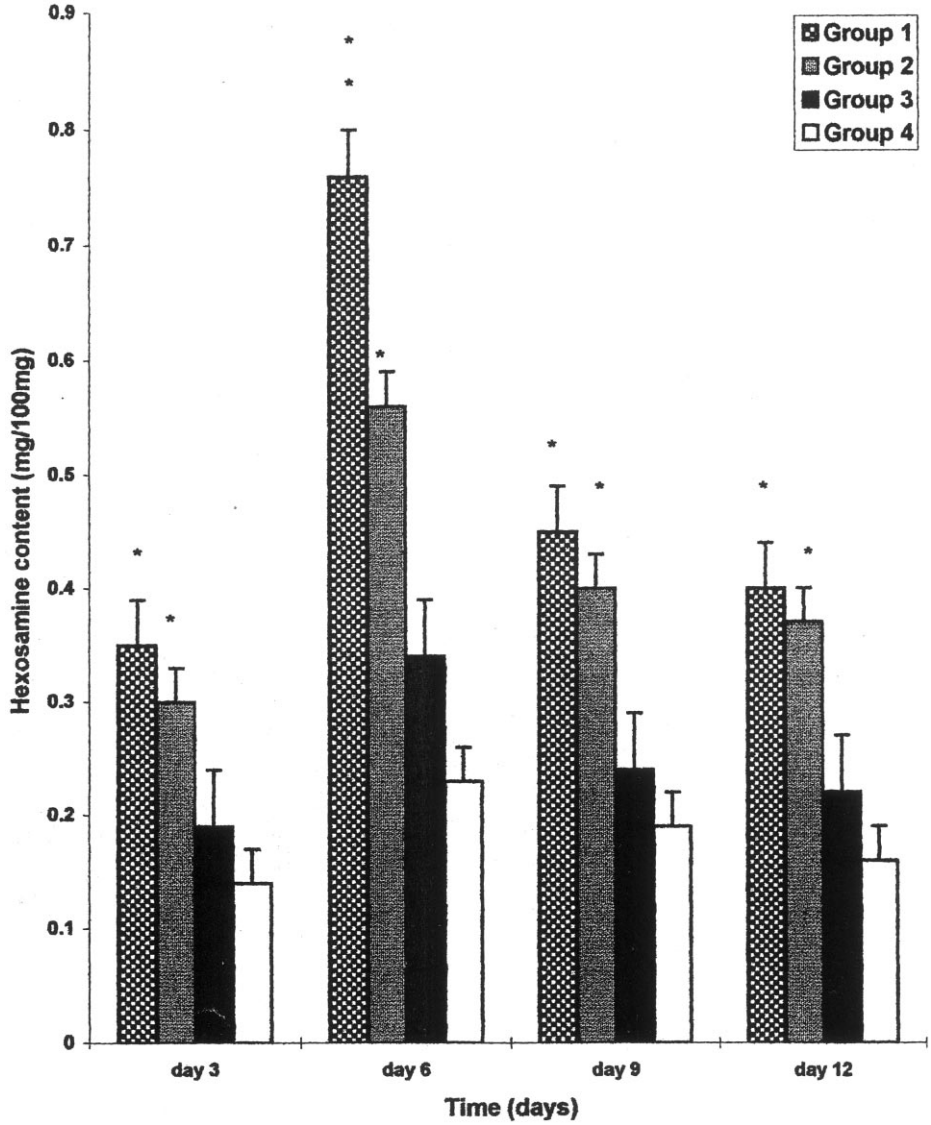

Fig. 2. Effect of different creams on the hexosamine content of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

In the present study, the effect of haruan on the biochemical components of the normal and diabetic healing wounds was analyzed. Uronic acid, protein, glycosaminoglycan and hexosamine levels were determined to establish the enhanced wound healing properties of haruan.

2. Materials and methods

2.1. Materials

Pentobarbitone sodium B.P. was supplied by Rhone Merieux (Ireland) Tallaght, Dublin.

Sprague Dawley (SD) rats were taken from the animal house at University Science Malaysia. Streptozotocin, papain, uronic acid, dermatan sulphate, methyl cellosolve and L-cystine were purchased from Sigma Chemical Co. St. Louis, MO. Haruan fish extract was bought from Major Interest Sdn Bhd. Cetrimide cream was purchased from Eupha Pharma (M) Sdn Bhd.

2.2. Methods

Formulated creams were analyzed for their wound healing properties on SD rats. Ninety-six male SD rats (250–300 g) were used in the study. The study was carried out in two parts: in one set of experiment, normal rats were used, while in the other, diabetic rats were used. Diabetes was induced in the same manner as described earlier (Chithra et al., 1998).

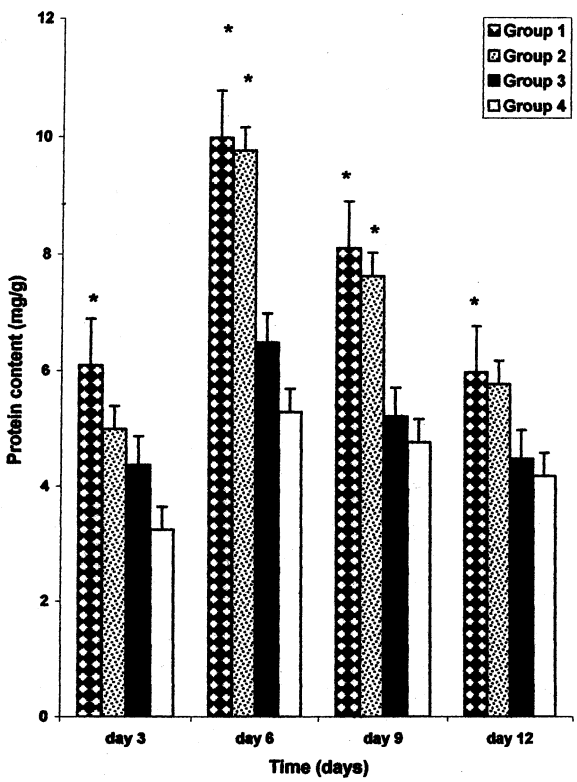

Fig. 3. Effect of different creams on the protein content of normal healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

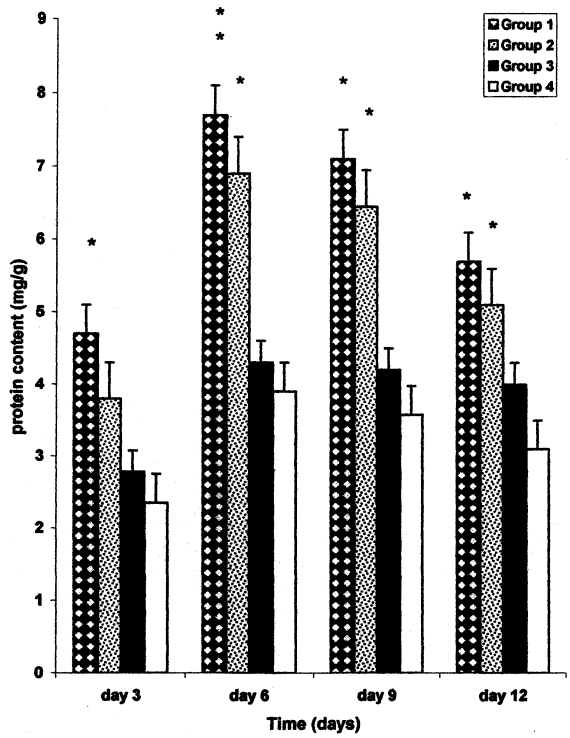

Fig. 4. Effect of different creams on the protein content of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

2.2.1. Wound creation

After the induction of diabetes, rats were anaesthetized with single shots of pentobarbitone sodium (60 mg/kg body weight) (Baie and Sheikh, 2000). Excision wounds were created in experimental rats. These wounds were used for the study of the biochemical components and the rate of wound contraction. All wounds were of full thickness type extending up to the adipose tissue. The right side of the back of each rat was lightly shaved and excision wounds of size 4 cm^2 were made by cutting out a 2 cm × 2 cm piece of skin from the shaven area.

2.2.2. Grouping of animals

After wound creation, experimental animals were divided into the following four groups in both sets of experiments (normal and diabetic rats):

Group 1: wounds treated with cream containing cetrimide + haruan extract
Group 2: wounds treated with cream containing haruan extract
Group 3: wounds treated with cetrimide cream
Group 4: wounds untreated (control group).

Each group consisted of 24 animals. The wounds were medicated with a local application (enough to cover the wound) of each cream. Rats were fed regular chow feed and had free access to water. Rats were sacrificed at intervals of 3, 6, 9 and 12 days after wound creation, and the entire wound was cut out and stored at −70°C until biochemical analysis.

2.2.3. Biochemical analysis of excision wounds

Biochemical analyses of excision wounds were done at intervals of 3, 6, 9 and 12 days of the wounding. Parameters involved in biochemical analysis included: uronic acid, hexosamine, wound contraction, glycosaminoglycan and protein content.

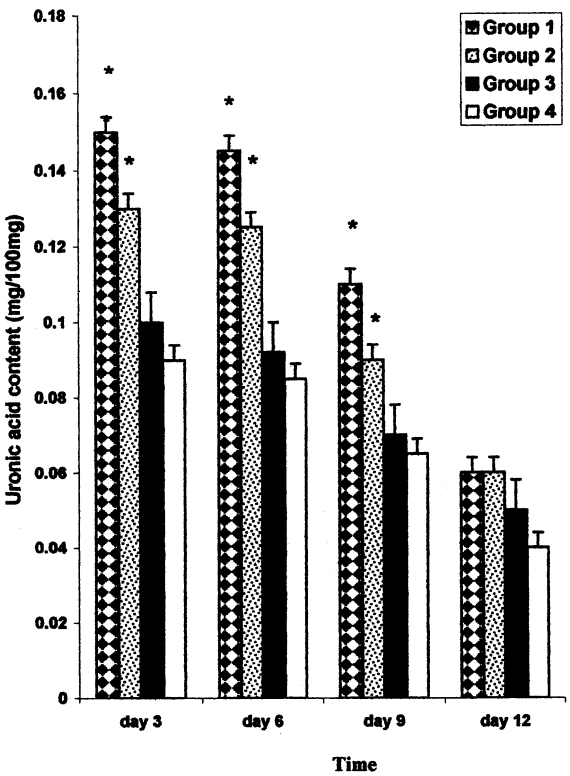

Fig. 5. Effect of different creams on the uronic acid content of normal healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

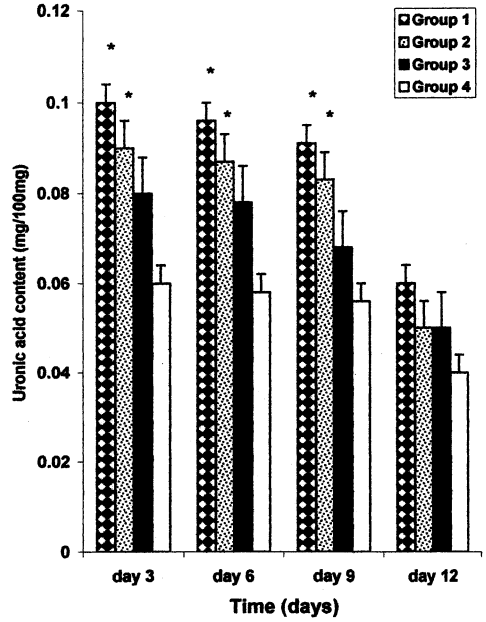

Fig. 6. Effect of different creams on the uronic acid content of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

2.2.3.1. Determination of hexosamines. The presence of hexosamines as constituents of glycoproteins was established very early. Both glucosamine and galactosamine appear to be present in serum glycoproteins. Recently, a new method for hexosamine analysis has been established by Isabel (1997), and was used in the present study and described below.

Wound ground tissue and glucosamine standards (0.05 mg) were dissolved in water in separate test tubes. To this mixture, was added 5 ml of 95% ethanol and mixed. The mixture was centrifuged for 15 min, decanted, suspended precipitate in 5 ml of 95% ethanol, centrifuged again and decanted. To the precipitated proteins, were added 2 ml of 3 N HCl, and hydrolyzed in a boiling water-bath for 4 h. Cooled the tubes and neutralized with 3 N NaOH until the solution was

barely alkaline and then diluted to 10 ml. with water. To 1 ml of diluted solution was added 1 ml of the freshly prepared acetyl acetone reagent and mixed. Blank solution was also prepared in the same manner. The tubes were tightly closed and placed in a boiling water-bath for 15 min. Cooled the tubes in tap water, added 5 ml of 95% ethanol and mixed. Finally, 1 ml of Ehrlich's reagent was added and mixed well, and diluted to 10 ml with 95% ethanol. The resultant purple color was stable for 3 h. The glucosamine contents for both sets of experiments (normal and diabetic) were determined spectrophotometrically using optical densities measurements at 530 μm. The results of hexosamine estimations of all formulations were statistically compared and probability (P) values were calculated.

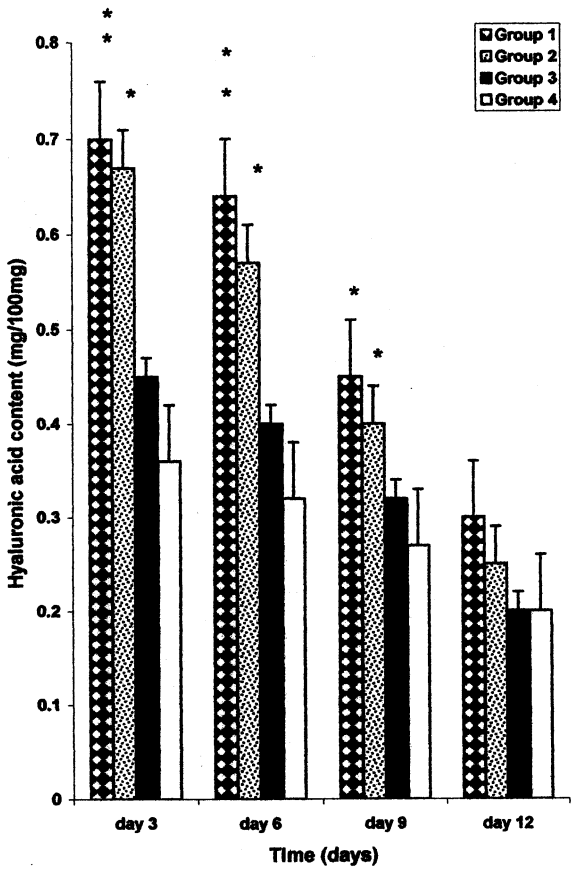

Fig. 7. Effect of different creams on the hyaluronic acid content of normal healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

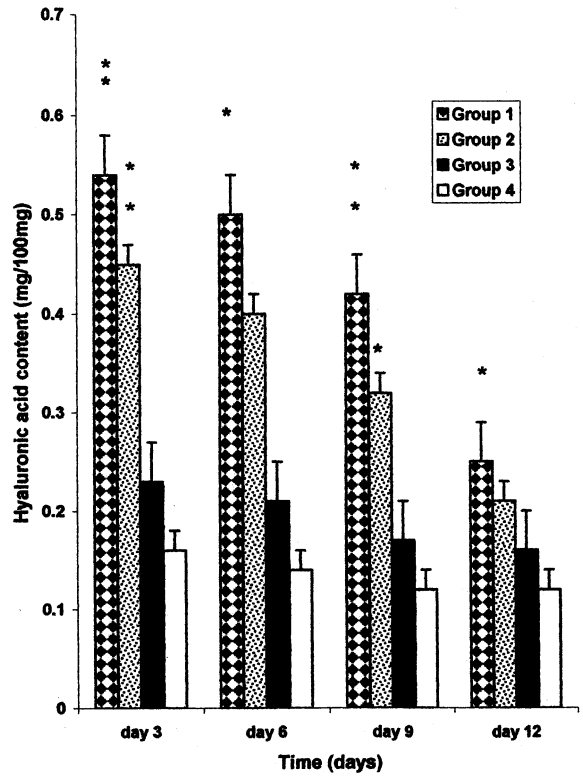

Fig. 8. Effect of different creams on the hyaluronic acid content of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

2.2.3.2. Protein determination. The protein content was determined as described by Lowry et al. (1951). For the estimation of total protein, wet granulation tissues (0.25 μg) were first extracted with TCA. Tissue was first homogenized in 5% TCA and centrifuged. The pallet was washed with 10% TCA, re-suspended in 5% TCA, and kept for 15 min in a water-bath at 90°C. The contents were centrifuged and the precipitated proteins were suspended in 5 μl of 8 N NaOH. The samples were mixed thoroughly by tapping and were covered with a rubber cap and left for 30 min. Fifty microliters of carbonate-copper solution was then added and mixed by tapping. After 10 min, 5 μl of diluted Folin Phenol Reagent was added with mixing and the samples were read spectrophoto-

metrically after 30 min at 530 μm. The results of all groups were statistically compared and *P* values were calculated.

2.2.3.3. Estimation of uronic acid. Uronic acid was extracted from the granulation tissue as described by Biter and Muir (1962). The tissue was digested with papain (10 mg/g wet weight of tissue) in 0.5 M acetate buffer, containing 0.005 M cysteine and 0.005 M di-sodium salt of EDTA at 65°C for 24 h. Five milliliters of sulfuric acid reagent was placed in a tube and cooled to 4°C. Then 1 ml of the sample was layered on the sulfuric acid. Tubes were closed with Teflon stoppers and were shaken gently with constant cooling. The tubes were then heated for 10 min in a boiling water-bath and cooled to room temperature. Then 0.2 ml of carbazol was added and tubes were shaken again, heated in boiling water-bath for 15 min and cooled to room temperature. The optical density was then read at 530 μm in a 1-cm cell spectroscopically. Results of all groups were statistically compared and *P* values were calculated.

2.2.3.4. Estimation of glycosaminoglycans (GAGs)

Extraction of GAGs. Total GAGs from the granulation tissue were extracted using the method described by Smith et al. (1980). Tissue samples (25 mg) were suspended in 1% SDS (sodium dodecyl sulfate) and heated in boiling water-bath for 10 min. After cooling to room temperature, Proteinase K (0.5 mg/ml) digestion was carried out at 60°C for 24 h. The digested

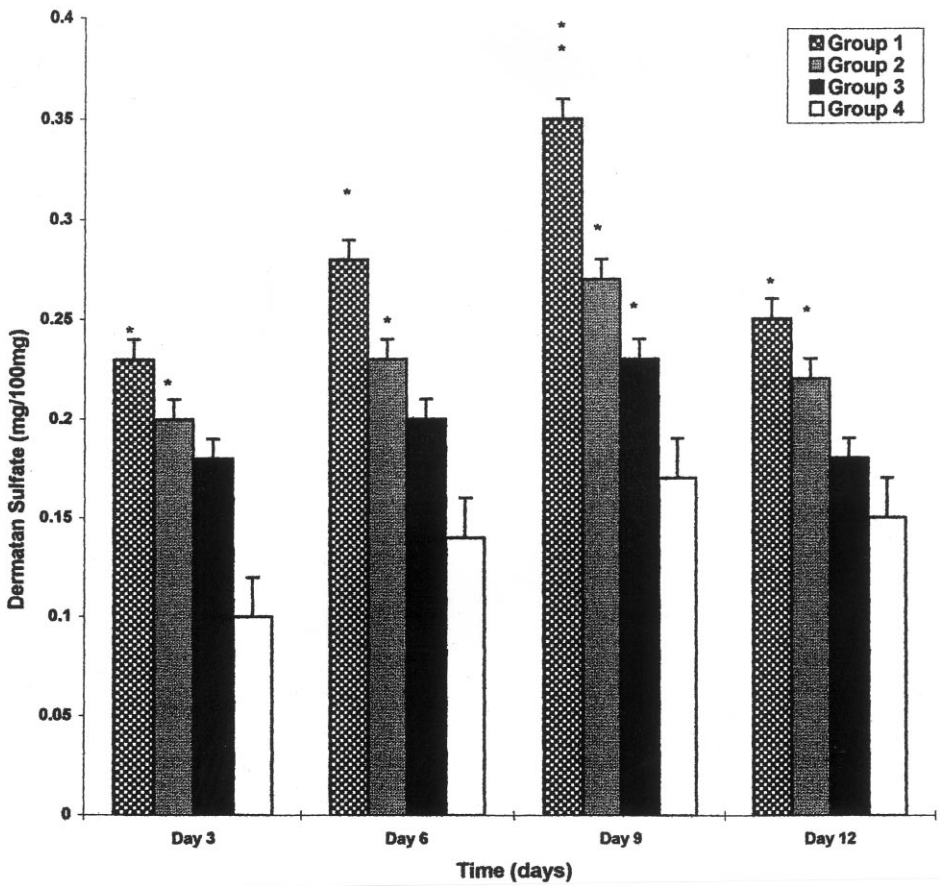

Fig. 9. Effect of different creams on the dermatan sulfate content of normal healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

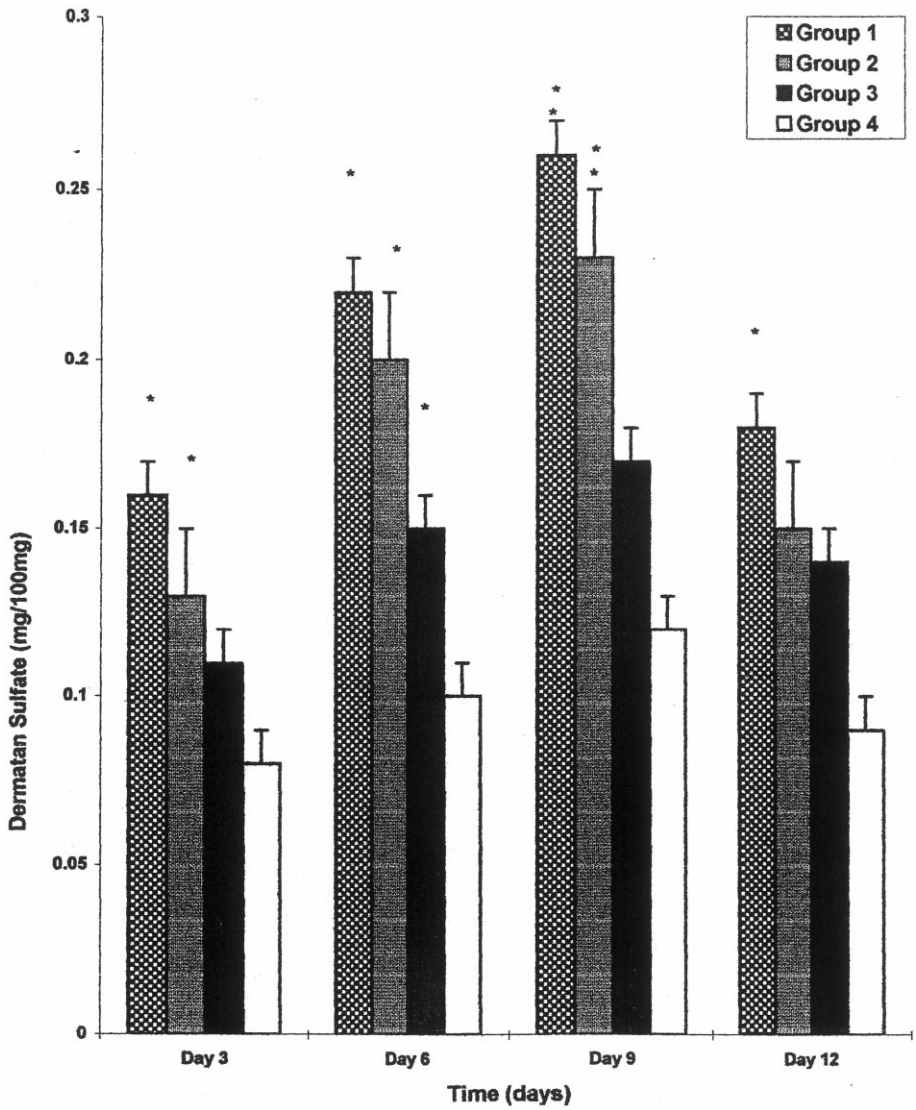

Fig. 10. Effect of different creams on the dermatan sulfate content of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

sample was precipitated with 10% TCA. The supernatant was extracted with chloroform/methanol (2:1). GAGs were extracted from the precipitated aqueous phase by addition of 5 ml of 95% ethanol saturated with 5% potassium acetate. The precipitates were dissolved in water and the amounts of GAGs were determined by the method of Chithra et al. (1998).

2.2.3.5. Determination of wound contraction. For the determination of wound contraction, excision wounds were traced on a transparent paper having a scale, and the change in wound size at various intervals was calculated as the percentage of wound area that had healed. The results of wound contraction studies of all groups were statistically compared and *P* values were calcu-

lated. The wound contraction percentage was determined using the following formula:

$$\%\ \text{Wound contraction} = \frac{\text{Healed area}}{\text{Total area}} \times 100$$

2.2.4. Statistical analysis

The results of different groups were compared statistically using two-way ANOVA. Statistically significant results are given as $*P < 0.05$ and $**P < 0.01$

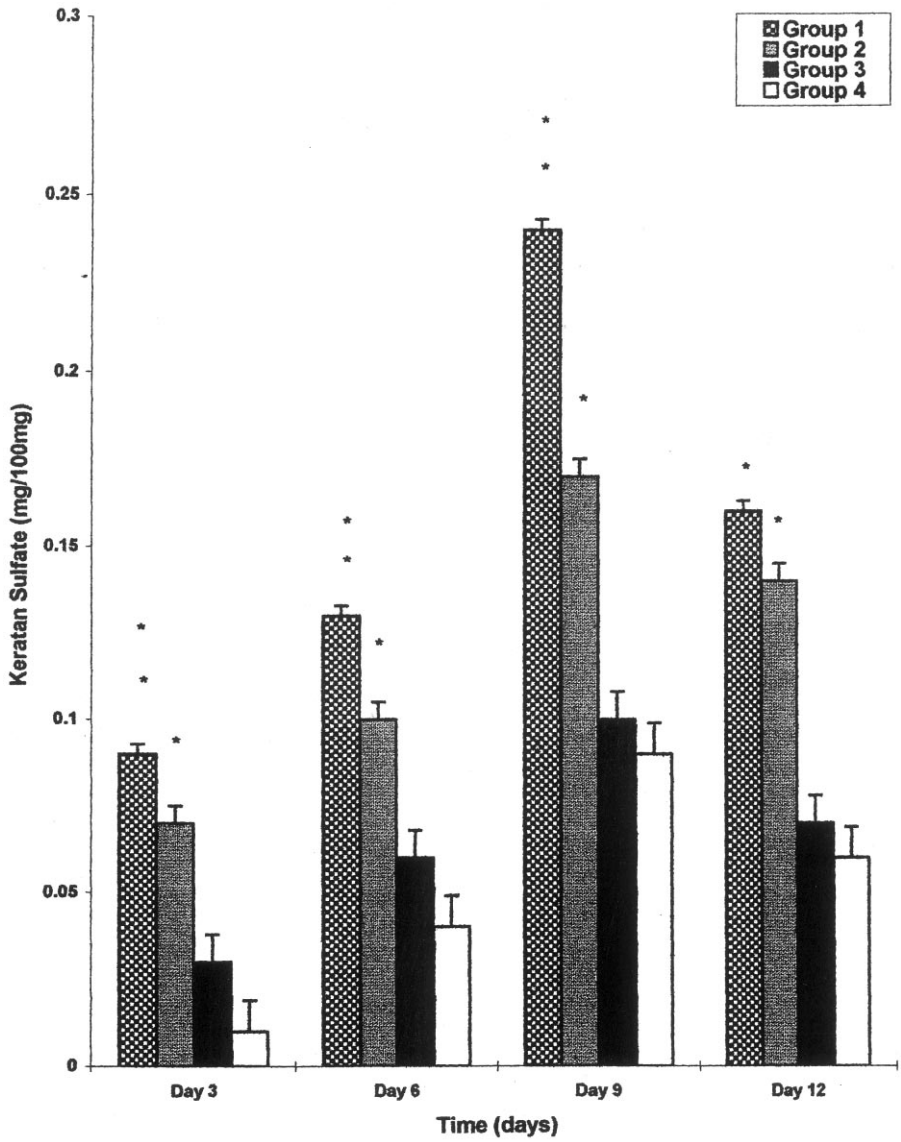

Fig. 11. Effect of different creams on the keratan sulfate content of normal healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

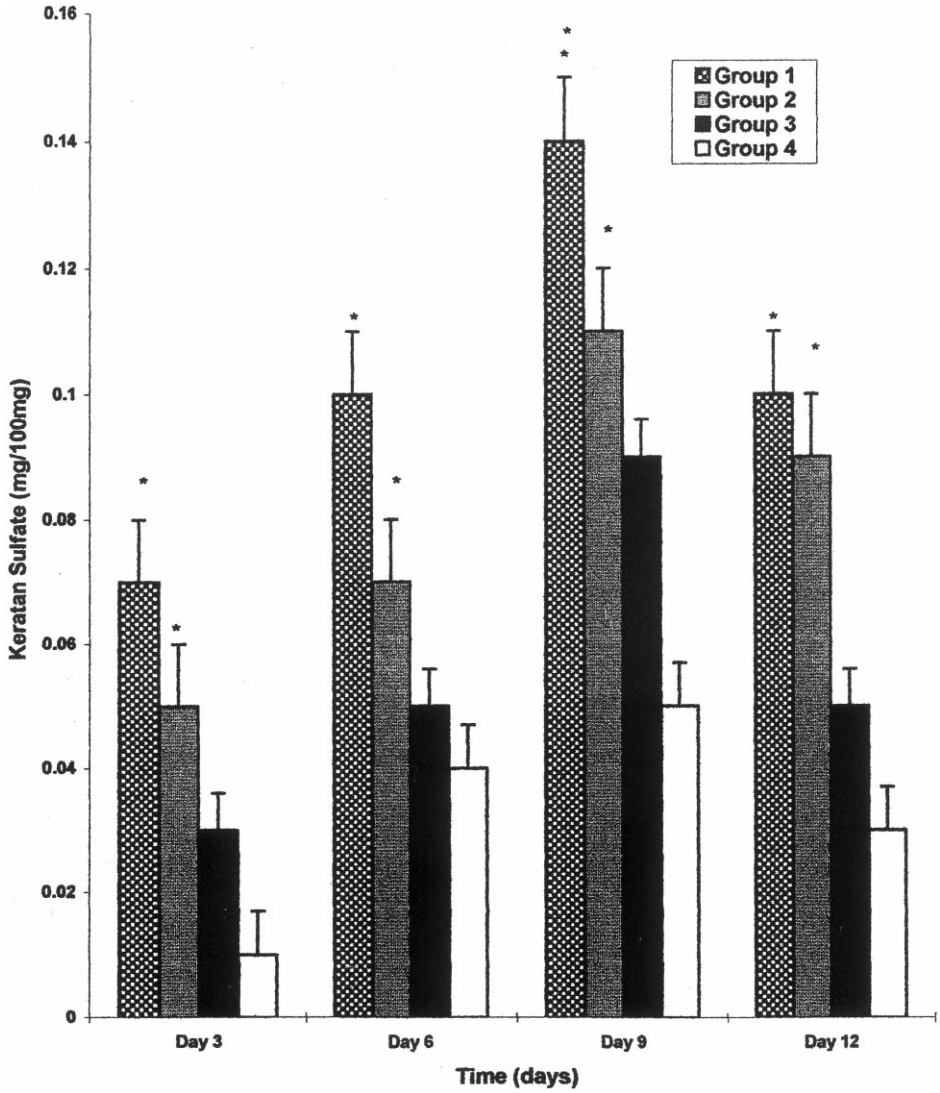

Fig. 12. Effect of different creams on the keratan sulfate content of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

3. Results

3.1. Biochemical analysis of excision wounds

Excision wounds were utilized to analyze the biochemical components of healing wounds. The biochemical components analyzed included collagen, hexosamines and glycosaminoglycans.

3.2. Hexosamine content determination

Hexosamine contents in the granulation tissue of all treated groups were analyzed and compared with the control group. It was observed that groups 1–3 had 70, 65 and 50% higher hexosamine contents, respectively (on day 6) in granulation tissue, when compared with the con-

trol group (Fig. 1). In diabetic rats, these levels were 60, 55 and 40% higher, respectively, for groups 1–3, when compared with control diabetic rats (Fig. 2). In all treated wounds, however, the hexosamine levels were found to decrease gradually and reached normal levels by day 12.

3.3. Determination of protein content

Protein contents of the granulation tissues of all treated groups in both sets of experiments are given in Figs. 3 and 4. There was a rapid increase in protein content in both sets of experiments and their levels reached to the maximum on day 6. After this time, their levels started decerasing. At all intervals, protein levels were higher in groups 1–3 as compared with group 4 (control).

3.4. Estimation of uronic acid

Uronic acid levels were determined at day 3, 6, 9 and 12. From Fig. 5, it can be seen that for

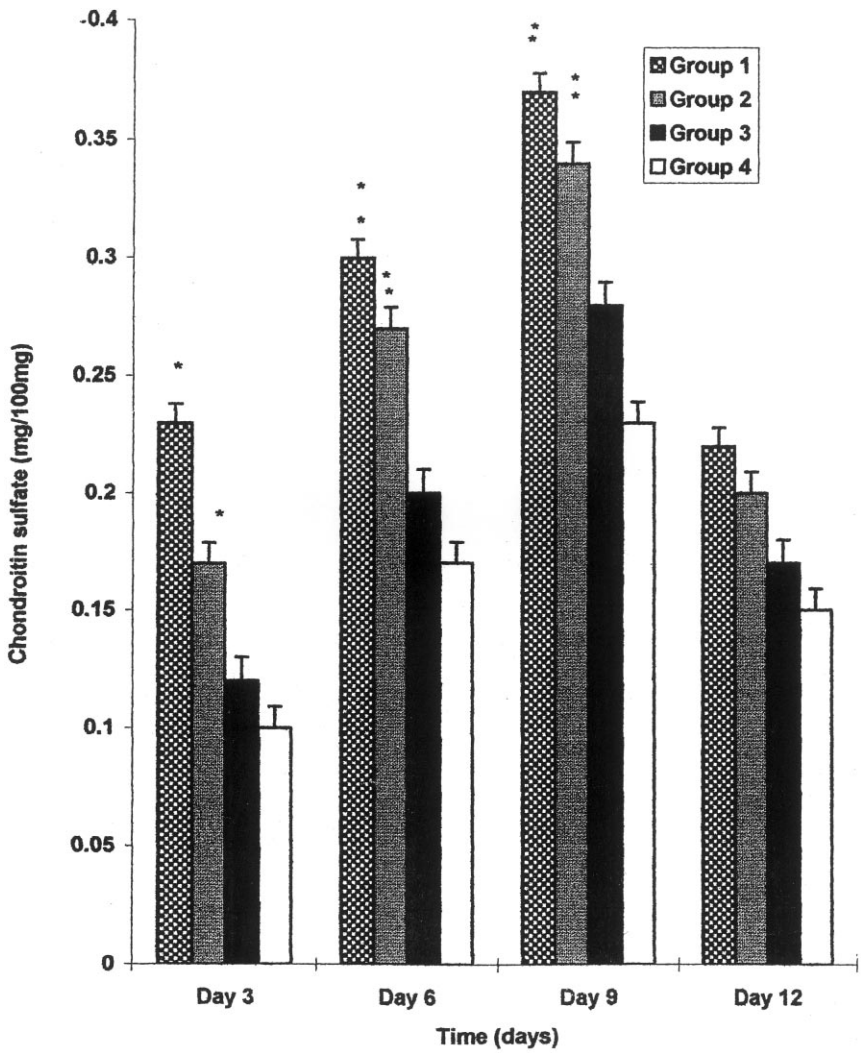

Fig. 13. Effect of different creams on the chondroitin sulfate content of normal healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

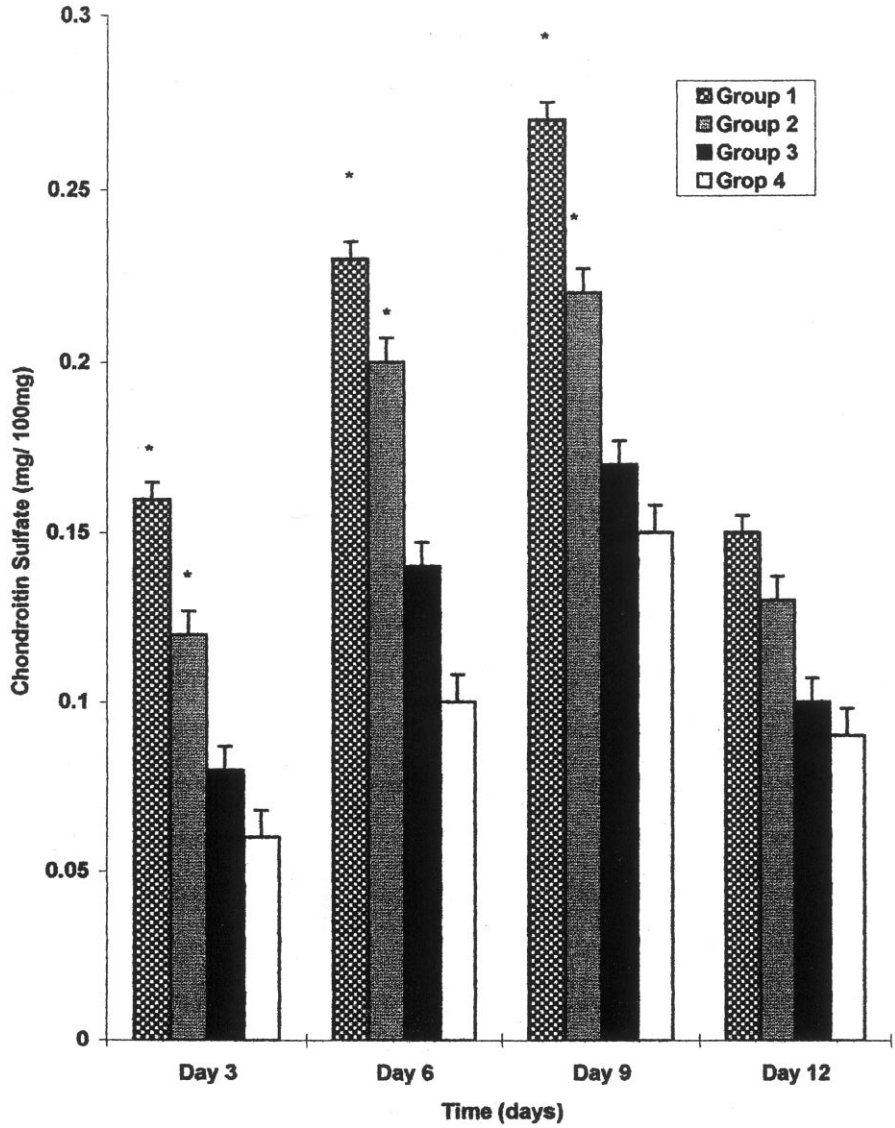

Fig. 14. Effect of different creams on the chondroitin sulfate content of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

normal rats, uronic acid content of groups 1 and 2 was highest on day 3 (75 and 65%, respectively), after which there was a constant decrease in their levels. Groups 1–3 gave higher values of uronic acid as compared to control group on all days. In diabetic rats, uronic acid synthesis followed the same sequence, but at slower rate (Fig. 6).

3.5. Determination of glycosaminoglycans

The relative amounts of various glycosaminoglycans present in the wound granulation tissues at various time intervals are shown in Figs. 7–14. From results, it is observed that hyaluronic acid formed the major Glycosaminoglycan in all

treated groups on day 3 and it was maximum in group 1 (70%) on day 3 (Fig. 7). Levels of other glycosaminoglycans (dermatan sulfate, keratan sulfate and chondroitin sulfate) were also found to be higher than the control group on day 3, 6, 9 and day 12 in normal rats as compared to diabetic rats (Figs. 8–14).

3.6. Estimation of wound contraction

Wound contraction in different groups is shown in Figs. 15 and 16. From results, wound contraction started immediately in all groups. Wound contraction was maximum in group 1 on day 9 (65%) in normal rats as compared to other groups

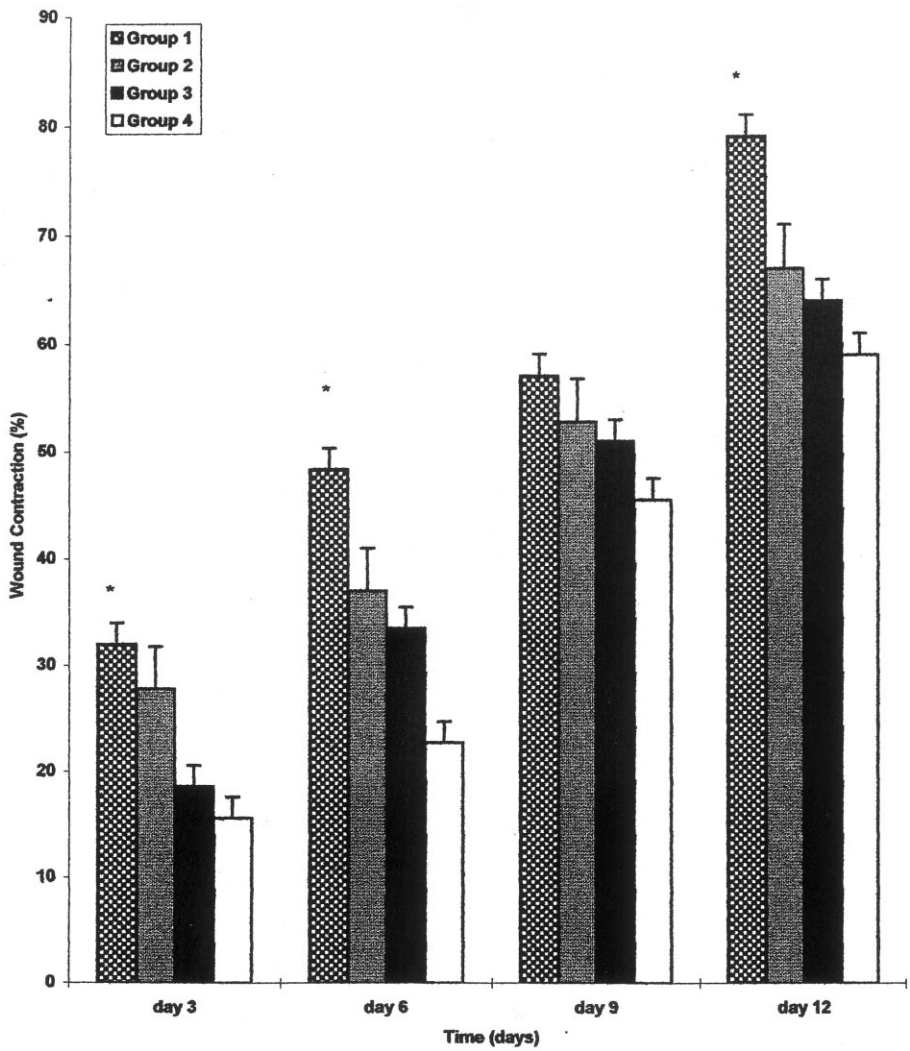

Fig. 15. Effect of different creams on the contraction of normal healing wounds at various time intervals. Results are presented as means $\pm$ S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

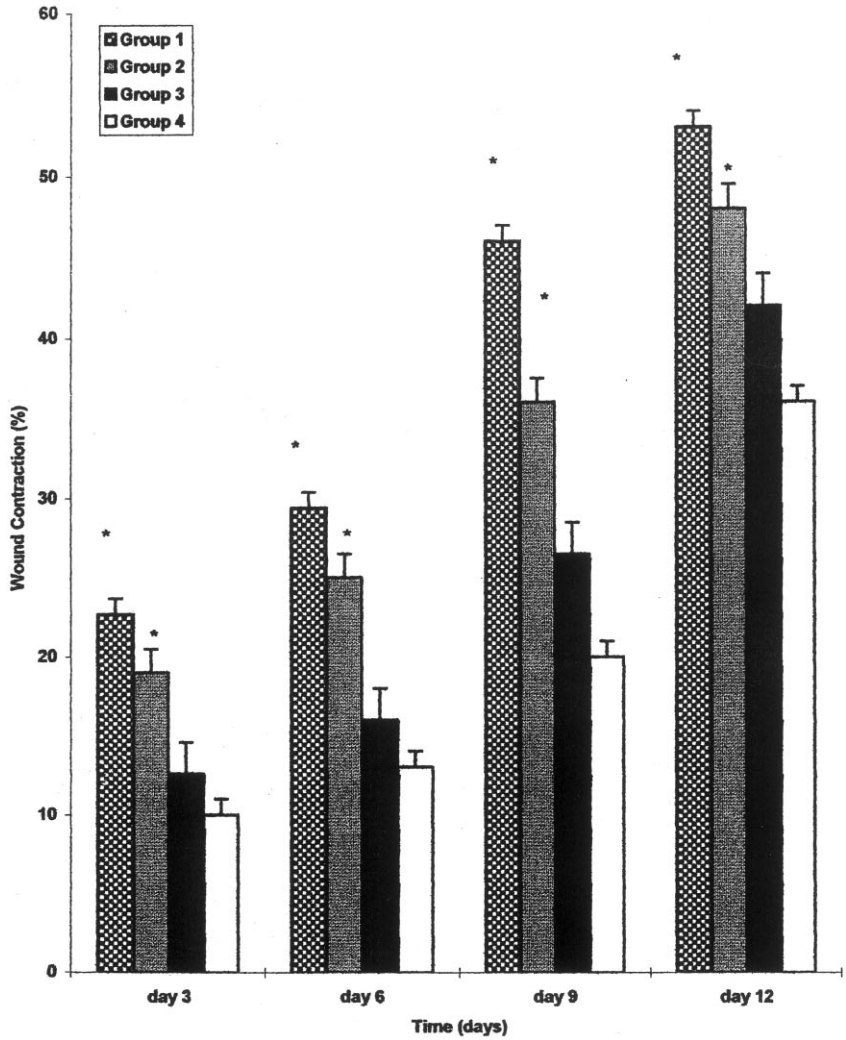

Fig. 16. Effect of different creams on the contraction of diabetic healing wounds at various time intervals. Results are presented as means ± S.D. Statistically significant data are given as $*P < 0.05$ and $**P < 0.01$.

($P < 0.05$). At day 6, wound contraction in all (normal) groups was significantly higher than diabetic rats ($P < 0.01$). Wound contraction on day 12, reached maximum in all treated groups.

4. Discussion

Hexosamine, sugar content of the wound, showed minimum levels in the early part of wound healing. There was an increase in the hexosamine levels with a decrease in the collagen syntheses which confirmed the enhancement of healing process (Dunphy and Udupa, 1955). It may be seen that the decrease in hexosamine content was associated with a concomitant increase in collagen content. The protein content of granulation tissues represents the protein levels and cellular proliferation (Chithra et al. 1998). Higher protein contents of groups 1 and 2 when compared with control group suggest that haruan treatment of wounds stimulate the cel-

leluar proliferation through an unknown mechanism.

GAGs are the important constituents of the extracellular matrix found on cell surfaces (D'Ardenne, 1992). They are also found free in the ground substance. The GAGs are the first components of the extracellular matrix to be synthesized during wound healing. In the present study, we have determined the types and amounts of the various Glycosaminoglycans (GAG) present in the granulation tissue of haruan treated and control (untreated) rat wounds. We have observed the significant changes in the GAG content. An increase in the uronic acid content of haruan treated wounds as compared to the control, suggests an enhanced synthesis of GAGs.

Hyaluronic acid has an important role to play in the early wound healing process. Earlier studies have shown that there is an increase in the hyaluronic acid content in early part of wound healing (Gallo and Bernfield, 1996). Changes in the hyaluronic acid levels affect cellular proliferation and the deposition of structural matrix (Toole, 1977). Hyaluronic acid has also been shown to stimulate collagen synthesis in fibroblast cultures (Mast et al., 1993). Fetal wounds which exhibit scarless wound healing have been found to contain 100% hyaluronic acid in the wound maritx in early part of wound healing (De Palma et al., 1989). The interaction of hyaluronic acid with keratinocytes also has an important role in the process of epithelialization (Okasala et al., 1995). Because hyaluronic acid may provide a more fluid that facilitates greater cell mobility and early remodeling. The increased amounts of hyaluronic acid in haruan treated wounds may result in the formation of a stable scar and may enhance the wound healing.

The increased amounts of dermatan, keratan and chondroitin sulfate in the haruan treated wounds also play an important role in the faster healing of wounds. dermatan sulfate is closely associated with collagen fibers (Fleischmajer et al., 1991). They have been shown to influence collagen fibril formation in vitro and may therefore contribute to the organization and strength of the fibrillar network in vivo (Scott, 1988). The haruan treated wounds might result in the improved wound healing in normal as well as diabetic rat wounds.

Haruan treated wounds also showed an increased rate of wound contraction, leading to quicker healing as confirmed by the increased healed area when compared to the control rat wounds. Haruan is known to contain ω-3 polyunsaturated fatty acids which can regulate prostaglandin synthesis and also can influence the immune system (Bowman and Rand, 1980). Haruan also contains the amino acids (glycine, aspartic acid and glutamic acid), required for faster wound healing (Mat Jais et al., 1994).

Cetrimide has long been believed to help in wound healing. Cetrimide was purchased and applied on the wounds to compare its healing properties with the two formulated creams (one containing haruan fish extract and other having cetrimide + haruan fish extract). It is clear from the results that wounds treated with cetrimide cream heal faster when compared with untreated wounds. There is a significant difference ($P < 0.01$) in wound healing effect of cetrimide when compared with two formulated creams (haraun and cetrimide + haruan cream). Statistical analysis showed that there is significant difference ($P < 0.05$) in the wound healing properties of groups treated with haruan cream and cetrimide + haruan cream. It is therefore concluded that cetrimide enhances the wound healing properties of haruan as cetrimide + haruan treated wounds show nigher values of protein, keratan sulfate, chondroitin sulfate and hexosamine content when compared with haruan treated wounds

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